Adenosine Diphosphate Translocation in Mitochondria. Nature of the Receptor Site for Carboxyatractyloside (Gummiferin)†

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ABSTRACT: Carboxyatractyloside (gummiferin, Gum), the 4-carboxylated precursor of atractyloside (Atr), is a noncompetitive inhibitor of the adenine nucleotide translocation in mitochondria. It does not affect the coupling mechanism in oxidative phosphorylation nor the ATPase activity in submitochondrial particles. Its high potency, its virtually immediate effect, and the noncompetitive character of its inhibition make carboxyatractyloside a better tool than atractyloside in kinetic studies of ADP or ATP transport by the inhibitor-stop technique. In the rat liver cell, mitochondria are the only subcellular particles to bind [^{35}S]carboxyatractyloside with high affinity (K_d = 5–10 nM) and high capacity (1.5–2 mol/mol of cytochrome *a*). The fractional saturation of mitochondria by increasing concentrations of Gum coincides with the fractional inhibition of the adenine nucleotide translocation. Carboxyatractyloside binds specifically to the inner membrane of mitochondria and is not displaced by ADP. Conversely, bound ADP is not displaced by carboxyatractyloside. Carboxyatractyloside binding is characterized by positive homotropic interactions. Lipid depletion or sonication of mitochondria abolishes these interactions, but the total number of binding sites remains the same. The protein nature of the mitochondrial receptor for carboxyatractyloside is assessed by the decrease of the carboxyatractyloside binding capacity upon heating, digestion with trypsin, or photooxidation. [^{35}S]Carboxyatractyloside binding to mitochondria is inhibited competitively by Atr and noncompetitively by bongkrekic acid. The inhibition of [^{35}S]carboxyatractyloside binding by bongkrekic acid is markedly enhanced by ADP. Release of [^{35}S]carboxyatractyloside previously bound to mitochondria is obtained by addition of carboxyatractyloside or of atractyloside but not by bongkrekic acid or ADP; however, when bongkrekic acid and ADP are added together, bound [^{35}S]carboxyatractyloside is released. Although isolated inner membrane vesicles obtained by hypotonic swelling of mitochondria can bind [^{35}S]carboxyatractyloside they display a lower affinity for this compound and a lower binding capacity than whole mitochondria. Full restoration of binding affinity and capacity is conferred by minute amounts of ADP or ATP (2 μM), but not by other nucleotides. Apocarboxyatractyloside (apo-Gum), which results from carboxyatractyloside by removal of the isovaleryl group, also inhibits the ADP translocation. However, in contrast to carboxyatractyloside, the inhibition caused by apocarboxyatractyloside is competitive with respect to ADP; furthermore, its binding to mitochondria does not display marked cooperative interactions and it is displaced by ADP. [^{35}S]Apocarboxyatractyloside binding is inhibited competitively by atractyloside, and noncompetitively by carboxyatractyloside and by bongkrekic acid.

Atractyloside (Atr[1]), a compound extracted from the thistle *Atractylis gummifera* because it may be readily obtained as [^{35}S]Atr of high specific radioactivity, has proven to be a useful tool in probing the adenylate carrier (Vignais *et al.*, 1970, 1971a; Klingenberg *et al.*, 1971, 1972). In a systematic exploration of Atr-related compounds, we have identified in extracts of *Atractylis gummifera* another powerful inhibitor of ADP translocation that we named gummiferin (Gum)[1] (Stanislas and Vignais, 1964). Gum was recently identified with 4-carboxy-Atr[2] (Figure 1). The evidence was based on the determination of the structure of carboxyatractyligenin (Danielli *et al.*, 1971, 1972) and on chemical data obtained with the whole glucoside molecule (Defaye *et al.*, 1971; Vignais *et al.*, 1971b). In the meantime, suggestive evidence for the identity of gummiferin and carboxyatractyloside was obtained by Luciani *et al.* (1971), who showed that carboxyatractyloside inhibited the ADP-stimulated respiration of mitochondria in the same way as gummiferin did (Stanislas and Vignais, 1964).

We have labeled Gum with ^{35}S to study its binding properties to mitochondria. This paper gives detailed evidence that, like Atr (Vignais and Vignais, 1970), Gum binds to the inner mitochondrial membrane and specifically inhibits adenine nucleotide translocation in mitochondria but, unlike Atr, it does it in a noncompetitive manner. Data are provided which show that the Gum sites are on the ADP translocase or in its neighborhood. Alterations of the binding properties of [^{35}S]-Gum brought about by the removal of mitochondrial phospholipids or the digestion of mitochondrial proteins or the chemical modification of the Gum structure are described and discussed as they relate to interactions of some specific groups of Gum (carboxylic and isovaleryl groups) with components of the Gum receptor site.

Experimental Procedure

Materials. [^{35}S]Atr and [^{35}S]Gum were extracted according to Wunschendorff and Braudel (1931) from the rhizomes

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[1] Abbreviations used are: Atr, atractyloside or atractylate; apo-Atr, apoatractyloside or apoatractylate; Gum, carboxyatractyloside or gummiferin; apo-Gum, apocarboxyatractyloside or apogummiferin; $APCH_2P$, adenosine 5′-methylenediphosphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

[2] Carboxyatractyloside marketed by Boehringer this fall has been found in our hands to have the same effects as gummiferin isolated in our laboratory.

of the thistle *Atractylis gummifera* that had been grown in the presence of [^{35}S]sulfate and separated by paper chromatography and electrophoresis (Stanislas and Vignais, 1964; Vignais and Vignais, 1971; Vignais *et al.*, 1971a). The whole procedure used to obtain [^{35}S]Gum consisted of the following.

EXTRACTION. Seedlings of *Atractylis gummifera* (700 g) that had been grown in the presence of [^{35}S]sulfate were grated, chopped, covered with distilled water (2.5 l.), and left to leach overnight in the cold. The pH was then brought to neutrality by small additions of calcium carbonate powder and the suspension heated at 60° for 20 min. The pieces of rhizomes were squeezed and the extract filtered. A stream of CO_2 was then bubbled for 1 hr through the filtrate and the slight precipitate formed removed by centrifugation. The supernatant fluid was evaporated to dryness under reduced pressure at 35°. The dry residue was extracted four times by refluxing in 500 ml of 85% ethanol for 30 min. After filtration, the ethanolic extract was concentrated to 15 ml by distillation under reduced pressure at 35° and eventually centrifuged to remove the remaining inulin.

PURIFICATION. Paper Chromatography. The concentrated extract (15 ml) was streaked on sheets of Whatman No. 3 paper (50 cm long) and run overnight by descending chromatography in the organic phase obtained by the mixture *n*-butyl alcohol–acetic acid–water (4:1:5, v/v). The [^{35}S]Gum and [^{35}S]Atr present in the extracts moved practically together, forming a large band ahead of slow moving pigments which could be eliminated. The band located by reference to markers and by radioactivity was cut out and eluted by water.

Paper Electrophoresis. The crude mixture of [^{35}S]Gum and [^{35}S]Atr eluted from the chromatograms and concentrated by freeze-drying was spotted on sheets of Whatman No. 3 paper (34 cm long) and submitted to high voltage electrophoresis (2500 V; 1 hr; solvent, pyridine–acetic acid–0.1 M EDTA (1.5:5:2500, v/v), in a refrigerated pherograph). The first electrophoretic run allows a further elimination of contaminating pigments. A large band containing both [^{35}S]-Gum and [^{35}S]Atr is cut out and eluted by water. From the second electrophoretic run the radioactive spots were located by autoradiography and the band containing [^{35}S]Gum and [^{35}S]Atr cut and eluted separately. The eluates were then systematically tested both for their biological activity and their radioactivity. The routine biological test is the oxygraphic assay described by Vignais *et al.* (1971b). It consists of the determination of (1) the amount of inhibitor bringing about 50% inhibition of the ADP-stimulated respiration of rat liver mitochondria (5 mg of protein); (2) the reversal (with Atr) or nonreversal (with Gum) of the inhibition of respiration by high ADP concentrations; and (3) the release of inhibition by uncouplers such as 2,4-dinitrophenol or FCCP.[1] This third point allows one to detect inhibitors of the respiratory chain if present as contaminants in the Gum preparation. The electrophoretic separation was repeated three–four times until the preparations of [^{35}S]Gum and [^{35}S]Atr were homogeneous as measured by a constant specific activity and checked by autoradiography. In the experiment described above where no particular care was taken to get quantitative recoveries, 70 mg of [^{35}S]Gum was obtained with a specific activity of 10^6 dpm/μmol.

The removal of the isovaleryl group at the C-2′ position of glucose gives a derivative of Gum called apo-Gum. apo-[^{35}S]Gum was obtained from [^{35}S]Gum as described by Piozzi *et al.* (1967) for the preparation of apo-Atr from Atr, except that anhydrous barium hydroxide in dry methanol was used. The chromatographic and electrophoretic behavior of [^{35}S]-Gum, [^{35}S]Atr, and of their labeled apo derivatives is illustrated in Figures 2A and 2B. The spots were detected by autoradiography. The specific radioactivity of [^{35}S]Gum and of its derivatives ranged from 0.3 to 1.2 $\times$ 10^6 dpm/μmol.

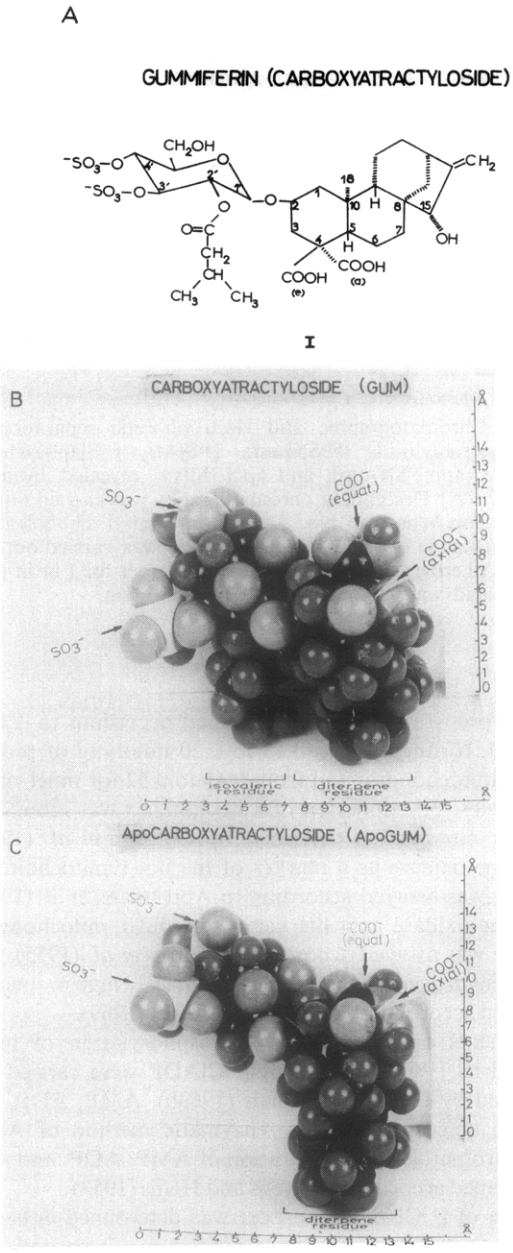

FIGURE 1: Structure (A) and space-filled model (B) of carboxyatractyloside (Gum). Note the bulky apolar portion of the molecule presented by the isovaleryl group and the diterpene moiety. (C) Space-filled model of apocarboxyatractyloside (apo-Gum).

Trypsin and phospholipase C from *Clostridium welchii* were purchased from Sigma Co. (St. Louis, Mo.).

Methods. Rat liver mitochondria were prepared from 0.27 M sucrose homogenates by the Hogeboom procedure and washed three times with 0.27 M sucrose, buffered with 1 mM Tris-HCl, pH 7.6. Inner membrane plus matrix and outer membrane particles of rat liver mitochondria were prepared following the procedure of Parsons *et al.* (1967) and inner membrane vesicles devoid of matrix were prepared according to a procedure developed in this laboratory (see Colbeau *et al.*, 1971). Protein concentration was measured by the biuret method. The cytochrome *a* content of rat liver mito-

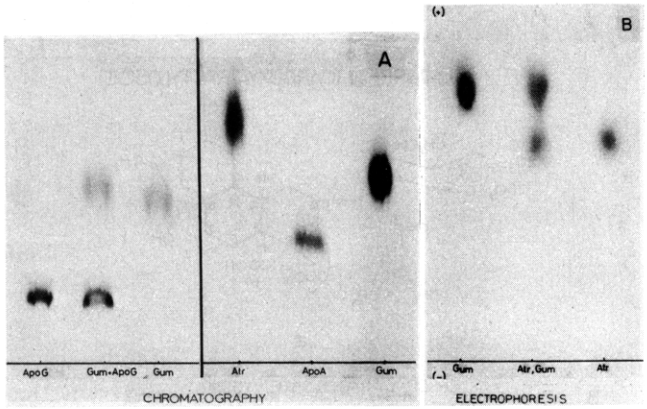

FIGURE 2: Chromatographic and electrophoretic separation of [^{35}S]carboxyatractyloside ([^{35}S]Gum), [^{35}S]Atr, [^{35}S]apocarboxyatractyloside (apo[^{35}S]Gum), and apo[^{35}S]Atr, revealed by autoradiography. (A) Descending chromatography was carried out for 18 hr at 20° on Whatman No. 3 paper in *n*-butyl alcohol–acetic acid–water (4:1:5, v/v). (B) Electrophoresis was carried out in a refrigerated pherograph on Whatman No. 3 paper for 1 hr in pyridine–acetic acid–water (0.65:2.5:1250, v/v), 66 V/cm.

TABLE I: Effect of Carboxyatractyloside (Gum) on the Dinitrophenol-Stimulated ATPase in Intact and Sonicated Mitochondria.[a]

Mitochondrial Preparation	Additions	P_i Released (nmol per min per mg of Protein) EDTA Medium	$MgCl_2$ Medium
Intact mitochondria (5 mg)	None	105	108
	0.12 μM Gum	97	89
	0.24 μM Gum	61	57
	0.48 μM Gum	7	35
	1.20 μM Gum	8	34
	1.20 μM Atr	76	51
Submitochondrial particles (2.2 mg)	None	25	580
	1.20 μM Gum	24	580
	1.20 μM Atr	25	580

[a] Submitochondrial particles were obtained from rat liver mitochondria by sonication in a Branson Sonifier for three periods of 30 sec at 9 kHz. After removal of the nondisrupted mitochondria by centrifugation at 20,000*g* for 5 min, the submitochondrial particles were sedimented at 100,000*g* for 30 min at 2°. Intact mitochondria and submitochondrial particles were incubated for 5 min at 25° in 2.4 ml of 0.110 M KCl, 0.01 M Tris-HCl, pH 7.4, 6 mM $MgCl_2$, or 0.1 mM EDTA, and 4×10^{-5} M dinitrophenol and 5 mM ATP. The incubation was ended by addition of 0.2 ml of 30% trichloroacetic acid. Inorganic phosphate was measured by the method of Fiske and Subbarow (1925).

chondrial preparations was determined according to Yonetani (1959); it ranged from 0.15 to 0.20 nmol/mg of protein for whole mitochondria and from 0.45 to 0.52 for inner membrane vesicles. Fractionation of mitochondria was monitored by marker enzymes as described by Colbeau *et al.* (1971). Cytochrome oxidase as a marker of the inner mitochondrial membrane was assayed according to Appelmans *et al.* (1955), monoamine oxidase as a marker of the outer mitochondrial membrane was assayed according to Tabor *et al.* (1955), and malate dehydrogenase as a mitochondrial matrix marker was assayed according to Englard and Siegel (1969).

Experiments on the oxidative phosphorylation of intramitochondrial [^{12}C]ADP and of [^{14}C]ADP were carried out as described by Duée and Vignais (1969b). AMP, ADP, and ATP were determined by the enzymatic method of Adam (1963). Chromatographic separation of AMP, ADP, and ATP was performed according to Krebs and Hems (1953).

The rate of [^{14}C]ADP transport was determined either by the direct or the back exchange procedure (Duée and Vignais, 1969a).

The photooxidation of mitochondrial particles was carried out in the presence of 10^{-5} M Rose Bengal with constant air bubbling as described by Westhead (1965). Lipid-depleted mitochondria were obtained essentially as described by Fleischer and Fleischer (1967) by extracting mitochondria with 10% water in acetone at 2°.

Binding of [^{35}S]Gum, [^{35}S]Atr or apo-[^{35}S]Gum to mitochondria or mitochondrial membranes was assayed in an isotonic saline medium made of 110 mM KCl, 10 mM Tris-sulfate, pH 7.2, and 0.1 mM EDTA or 6 mM $MgCl_2$. The EDTA or $MgCl_2$ medium can both be used for [^{35}S]Gum binding to whole mitochondria but the presence of $MgCl_2$ favors [^{35}S]Atr binding to isolated inner membrane vesicles. The $MgCl_2$ medium was therefore often used for assaying [^{35}S]Gum binding relative to [^{35}S]Atr binding; however, when ADP was used, EDTA was preferred to lower the adenylate kinase activity. The final protein concentration ranged from 0.3 to 0.9 mg/ml, except for the experiment described in Table III in which 2 mg/ml were used. The assay was carried out in a series of tubes with increasing concentrations of the labeled inhibitor. The total volume per tube was usually 10 ml. After an incubation at 2° for 45 min, the particles were sedimented by high speed centrifugation. The inside walls of the centrifuge tubes were rinsed with the saline solution and carefully blotted with filter paper strips. The pellets were then solubilized in 1 ml of formamide at 180° and the radioactivity was determined by liquid scintillation.

Results

Inhibition of ADP and ATP Dependent Reactions in Mitochondria. Figure 3 shows that 5 μM Gum totally inhibits the oxidative phosphorylation of [^{14}C]ADP added to mitochondria (Figure 3A) but only slightly affects the kinetics of phosphorylation of the internal [^{12}C]ADP (Figure 3B). The plateau phase in the synthesis of internal ATP, which follows the rapid initial phosphorylation and lasts from the first to the second minute of incubation was slightly smaller in the absence of Gum. This might be due to the occurrence in the mitochondrial suspension of a small fraction of internal ADP which had leaked out of the matrix space and therefore behaves as external ADP. It was found that this leakage is not prevented by Gum (unpublished results).

Conversion of internal [^{12}C]AMP into [^{12}C]ATP was Gum insensitive. Similar results had been obtained with Atr (Duée and Vignais, 1969b) and interpreted as the result of

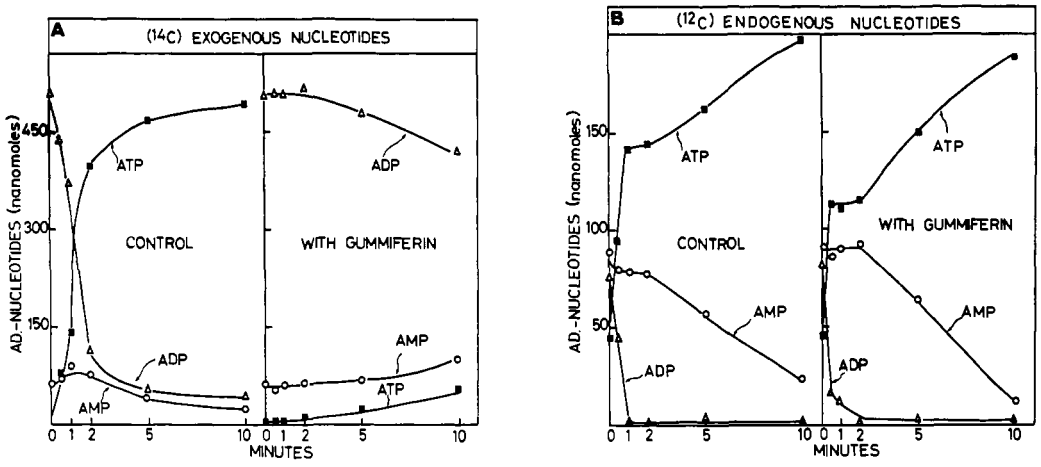

FIGURE 3: Effect of carboxyatractyloside (Gum) on the oxidative phosphorylation of (B) internal and (A) external adenine nucleotides. Mitochondria (17 mg) were incubated at 2° in 110 mM KCl, 10 mM phosphate buffer, 0.1 mM EDTA, 0.5 mM [^{14}C]ADP, final pH 7.4, and, when present, 5 μM Gum in a total volume of 4 ml. The incubation was terminated by addition of 0.3 ml of 30% perchloric acid. The total amount (^{12}C + ^{14}C) of each nucleotide AMP, ADP, and ATP was determined enzymatically. In a parallel assay the adenine nucleotides present in the perchloric extract were separated by paper chromatography and located under ultraviolet light; their respective areas were cut out and their ^{14}C content measured with a gas flow counter.

two coupled reactions, namely an ATP-dependent transphosphorylation of AMP into ADP and the oxidative phosphorylation of ADP into ATP; the transphosphorylation of AMP into ADP was supposed to involve the combined effect of a GTP–AMP phosphotransferase and of nucleoside diphosphokinase located inside the "ATR barrier," *i.e.*, on the matrix side of the inner membrane.

The inhibition of the entry of ATP into mitochondria by Gum is illustrated in Figure 4. The incubation medium contained mersalyl, an inhibitor of phosphate transport (Fonyo, 1968; Tyler, 1969) and FCCP,[1] an uncoupler of oxidative phosphorylation. Under these conditions, ATP after entering mitochondria was hydrolyzed into ADP and phosphate. Accumulation of phosphate in the matrix space, due to the inhibition of the phosphate carrier by mersalyl, resulted in a swelling whose rate and extent reflected the degree of inhibition of the efflux of phosphate from mitochondria. By inhibiting the ATP entry, Gum prevented the ATP-induced swelling (Figure 4A). Gum was more efficient than Atr and in contrast to Atr (Figure 4B) the inhibition caused by Gum was not relieved by increasing the ATP concentration (Figures 4C and 4D).

Gum did not inhibit the dinitrophenol-stimulated hydrolysis of ATP by sonicated mitochondrial particles which are devoid of permeability barrier toward adenine nucleotides (Souverijn *et al.*, 1970), whereas it strongly inhibited the ATPase activity of whole mitochondria (Table I). This observation rules out a direct effect of Gum on the mitochondrial ATPase activity.

Inhibition of ADP Transport in Mitochondria. The above results obviously point to specific inhibition by Gum of adenine nucleotide transport in mitochondria. Double reciprocal plot data in Figure 5A directly demonstrate that the mitochondrial ADP transport is inhibited by Gum and that the inhibition is noncompetitive. This may be contrasted with the apparently competitive inhibition caused by Atr (Figure 5B). In this case the departure of plots from linearity is probably due, as discussed for other tightly bound inhibitors by Henderson (1972), to the tight binding of Atr to mitochondrial membranes and to the large variation of the free Atr concentration when the Atr concentration is fixed and that of ADP varies.

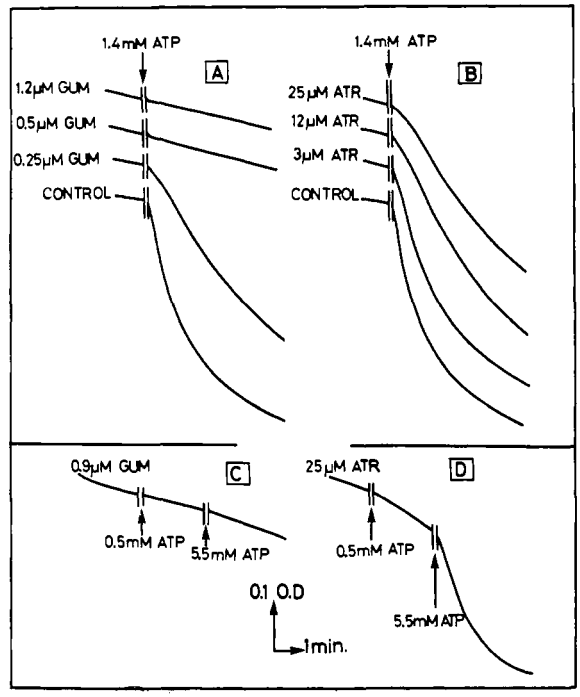

FIGURE 4: Inhibition by carboxyatractyloside (Gum) and Atr of ATP entry into mitochondria. Rat liver mitochondria (1.6 mg) were incubated at 25° in 3 ml of 0.110 M KCl, 0.02 M Tris-HCl, pH 7.4, 10^{-5} M rotenone, 0.5 mM EDTA, 25 μM mersalyl, 2 μM FCCP, and Gum or Atr as indicated. Swelling was induced by addition of ATP and followed by the decrease of absorbancy at 546 nm in an Eppendorf photometer. The initial absorbancy was about 0.650.

Inhibition of ADP translocation by Gum has also been observed for rat heart mitochondria (not shown here) and for yeast mitochondria (Lauquin, 1972).

The inhibition of the ADP translocation caused by Gum does not involve a significant lag time. In fact, as shown in

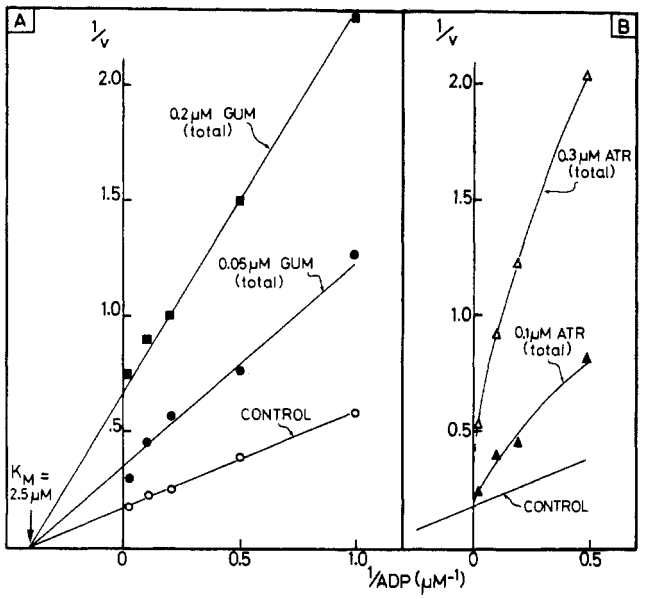

FIGURE 5: Inhibition of ADP translocation by carboxyatractyloside (Gum) and Atr. Rat liver mitochondria (300 mg of protein in 10 ml of 0.27 M sucrose) were preloaded with [^{14}C]ADP by incubation with 30 μM [^{14}C]ADP for 15 min at 4°; after two successive washings with 0.27 M sucrose, they were resuspended in 0.27 M sucrose. Aliquots (150 μl) of the suspension of ^{14}C-labeled mitochondria, containing 3 mg of protein, were incubated in 5 ml of the standard EDTA medium in the presence of different concentrations of inhibitors for 1 min at 2°. The back-exchange was initiated by the addition of different concentrations of ADP ranging from 1 to 50 μM. The exchange was ended, after a period of 20 sec at 2°, by rapid filtration (2 sec) through a Millipore filter (HAWP 0.45 μ) of a 0.5-ml aliquot and the radioactivity of the filtrate was determined by liquid scintillation. The rate of translocation v is expressed in nanomoles of [^{14}C]ADP exchanged per minute per milligram of protein. It is to be noted that the concentration of inhibitors indicated on the figure corresponds to the *total* amount of inhibitor used and not to the amount of *free* inhibitor.

TABLE II: Use of Carboxyatractyloside (Gum) to Stop the ADP Translocation in Mitochondria.[a]

Sequence of Additions	v (nmol of ADP per min per mg of Protein)
100 μM [^{14}C]ADP, 30 sec, then 4 μM Gum, 5 sec, followed by rapid centrifugation	6.9
Simultaneous addition of 100 μM [^{14}C]-ADP and 4 μM Gum, 5 sec, followed by rapid centrifugation	0.2
4 μM Gum, 5 sec, followed by 100 μM ADP, 30 sec, and then rapid centrifugation	0.01

[a] Rat liver mitochondria (8.8 mg of protein) were incubated in 5 ml of the standard EDTA medium at 2° with [^{14}C]ADP and Gum. The amount of [^{14}C]ADP incorporated in the matrix space was calculated from the amount of [^{14}C]ADP present in the mitochondrial pellet recovered by centrifugation of the suspension, after correction for the [^{14}C]ADP in the sucrose space.

Table II, either the preincubation of mitochondria with Gum followed by addition of ADP or the simultaneous addition of ADP and Gum to mitochondria results in only a negligible incorporation of [^{14}C]ADP compared to the control test. For this reason Gum is a convenient tool for terminating ADP or ATP transport in mitochondria. Since its inhibitory effect does not depend on the nucleotide concentration, it is more suitable than Atr, which was used in earlier studies by Vignais and Duée (1966) and subsequently by Pfaff and Klingenberg (1968) and Duée and Vignais (1969a). The [^{14}C]ADP incorporation into the matrix space is usually estimated from the radioactivity of the pellet of mitochondria, after correction for the [^{14}C]ADP present in the extra-matrix space. A parallel incubation with [^{14}C]sucrose usually is carried out to determine the volume of the extra-matrix space. The extra-matrix space can be more easily determined from the radioactivity of the mitochondrial pellet when [^{14}C]ADP is added after Gum since, as shown in Table II (third experiment), Gum prevents [^{14}C]ADP from entering the matrix space.

Affinity of Gum for the Inner Mitochondrial Membrane. Preliminary tests on [^{35}S]Gum binding to isolated subcellular particles from rat liver had shown that mitochondria are the only particles to bind Gum with high affinity and capacity. For instance, a crude microsomal fraction obtained by centrifugation of a postmitochondrial supernatant between 20,000 and 100,000g was found to bind less than 5 pmol of [^{35}S]Gum/mg of protein (with a K_d of 10 nM) compared to about 300 pmol in the case of mitochondria (K_d = 6 nM). Most probably binding of [^{35}S]Gum to crude microsomes was due to the presence of contaminant mitochondrial fragments.

To elucidate which compartment of mitochondria binds [^{35}S]Gum preferentially, mitochondria were incubated with [^{35}S]Gum for 30 min at 2°, to allow complete equilibration. They were then sedimented by centrifugation, and fractionated into outer membrane, inner membrane plus matrix, and inner membrane particles, taking as marker enzymes monoamine oxidase for the outer membrane, cytochrome oxidase for the inner membrane, and malate dehydrogenase for the matrix space. Data in Table III indicate that the amount of [^{35}S]-Gum bound per milligram of membrane protein is the highest in the inner membrane and the lowest in the outer membrane. A similar conclusion has been reported for [^{35}S]-Atr binding (Vignais and Vignais, 1970). Only traces of [^{35}S]Gum were found in the supernatant fluid recovered after centrifugation of the crude inner and outer membrane fractions as well as in the matrix fluid. Confirmatory evidence for the selective affinity of [^{35}S]Gum toward the inner mitochondrial membrane has been obtained in direct binding tests using purified inner and outer mitochondrial membranes (see below).

Properties of [^{35}S]Gum Binding to the Inner Mitochondrial Membrane either in Situ or after Isolation. Binding of gum to mitochondria is a rapid process. It is complete in less than 2 min at 2°. The binding experiments described here lasted for periods between 30 and 45 min and therefore corresponded to conditions of equilibrium between bound and free Gum. Furthermore, it has been checked (see below) that the molecule of Gum is not modified upon binding to mitochondria.

As shown previously (Vignais *et al.*, 1971b), in plotting the amount of [^{35}S]Gum bound at pH 7.2 against the concentration of free [^{35}S]Gum, a sigmoid-shaped curve with a well-defined saturation plateau is obtained suggesting that Gum

TABLE III: Distribution of Bound [^{35}S]Carboxyatractyloside ([^{35}S]Gum) in Submitochondrial Fractions.[a]

Fraction	Total Protein (mg)	Bound [^{35}S]Gum: Protein (pmol/mg)	Bound [^{35}S]Gum: Total (nmol)	Cytochrome Oxidase[b]	Monoamine Oxidase[c]	Malate Dehydrogenase[d]
Mitochondria	835	320	267	1.4	5.4	1.7
Low-speed pellet	458 (58)	420	204 (76)	1.7 (70)	5.3 (58)	1.8 (62)
High-speed pellet	15 (2)	185	3 (1)	0.9 (1)	47 (16)	0.3 (<1)
Supernatant	210 (25)	10	3 (1)	<0.1		0.9 (13)
Inner memb + matrix		435		1.8	3.8	2.0
Inner memb		730		3.4	4.9	0.8
Matrix		<40				2.3
Outer memb		152		0.7	60	0.1

[a] Rat liver mitochondria (1 g of protein) were incubated in 500 ml of the standard $MgCl_2$ medium and 1 μmol of [^{35}S]Gum. After 30 min of incubation at 2°, the mitochondria were collected by centrifugation at 15,000g for 10 min (835 mg of protein), resuspended in 400 ml of 20 mM phosphate buffer, pH 7.4 (from which solution aliquots were taken for analysis), and allowed to swell for 30 min at 2°. Three subfractions were subsequently obtained: a crude inner membrane + matrix fraction (low-speed pellet) collected by centrifugation at 1900g × 15 min, a crude outer membrane fraction (high-speed pellet) spun down at 35,000g × 20 min, and the remaining supernatant fluid. The two crude membrane fractions were further purified as described in Methods but relatively large losses made it impossible to draw a balance sheet of the recovery. [b] Micromoles of cytochrome c oxidized per min per mg of protein. [c] Nanomoles of benzaldehyde formed per min per mg of protein. [d] Micromoles of NADH formed per min per mg of protein. Per cent recovery is shown in parentheses.

binds to mitochondria with cooperative interactions up to saturation. The inhibitory effect of Gum on the ADP translocation was compared to the fractional saturation of the Gum sites. It was found that the binding curve and the inhibition curve are superimposable, indicating that the inhibition of the ADP translocation by Gum is directly related to Gum binding. At pH 7.2, the amount of Gum bound at saturation ranged from 1.5 to 2 mol/mol of cytochrome a in rat liver mitochondria and from 2.3 to 2.8 in rat heart mitochondria with a K_d value between 5 and 10 nM. In the usual test conditions the saturation plateau is attained at 0.2–0.3 μM [^{35}S]-Gum, and holds for concentrations of total [^{35}S]Gum up to 2 μM. However, at much higher concentrations (for instance, 6 μM) additional binding sites of lower affinity are revealed. The sigmoidicity of the curve is observable with freshly isolated mitochondria and is lost upon aging. ADP does not modify the sigmoidicity of the Gum binding curve in fresh mitochondria but it can restore it in aged mitochondria. Those experiments were concluded by testing the effect of ions and pH on Gum binding. The binding capacity and affinity of whole mitochondria for [^{35}S]Gum were virtually the same in a pH range of 6–8.5 and in the three following media: (a) 0.27 M sucrose buffered with 10 mM Tris-sulfate, pH 7.2; (b) 0.11 M KCl–6 mM $MgCl_2$–10 mM Tris-sulfate, pH 7.2; (c) 0.11 M KCl–0.1 mM EDTA–10 mM Tris-sulfate, pH 7.2.

Below pH 6, there was a loss in the cooperative binding of Gum and a disappearance of the saturation plateau which was replaced by a slope corresponding to a new set of weak sites. Although the acidic pH conditions used are far from being physiological, the appearance of low affinity binding sites is noteworthy in view of the pK values of the carboxylic groups of Gum (pK_1 = 3.4 and pK_2 = 6.7; unpublished results). An increase of the amount of low affinity sites has also been reported (Vignais *et al.*, 1970) for binding of Atr at a pH lower than 5 (the pK of the carboxylic group of Atr is 5.7). Most likely the increased binding in both cases (Gum and Atr) was due to protonation of carboxylic groups. As discussed later (*cf.* the Discussion) the second Gum carboxylic group in the un-ionized form (below pH 6) may participate in internal H bonding, bringing the Gum molecule into a conformation which may facilitate its solubilization into the lipid phase of the mitochondrial membrane.

The ability of the inner and outer mitochondrial membranes to bind [^{35}S]Gum was tested after separation of both membranes from rat liver mitochondria. Isolated inner membrane but not outer membrane displayed affinity for [^{35}S]Gum (Figure 6). However, when calculated on the basis of the cytochrome a content (taken as a specific component of the inner membrane) two–four times less [^{35}S]Gum was found to bind at saturation to isolated inner membrane than to whole mitochondria. Furthermore, better binding affinity and capacity was obtained with the EDTA medium than with the $MgCl_2$ medium; this contrasts with the lack of effect of EDTA or $MgCl_2$ on the Gum binding in whole mitochondria. Addition of ADP together with Gum in the incubation medium restored the full ability of the inner membrane to bind [^{35}S]-Gum. The restoring effect of ADP is shared by ATP and $APCH_2P$ but not by UDP, CDP, GDP, and AMP. A similar nucleotide specificity has been reported for the adenine nucleotide translocation in mitochondria (Pfaff and Klingenberg, 1968; Winkler *et al.*, 1968; Duée and Vignais, 1969a). The maximal restoring effect is observed at 2 μM ADP or ATP and the half-maximal effect at less than 0.1 μM ADP or ATP. This increase of bound Gum upon addition of ADP or ATP to inner membrane vesicles recalls a similar phenomenon already reported for Atr binding (Vignais and Vignais, 1971; Vignais *et al.*, 1971b). At the present time it is not known whether the ADP- or ATP-induced capacity of isolated inner mitochondrial membrane to bind [^{35}S]Gum is due to a restoration of deteriorated sites or to an unmasking of sites which may have been buried due to membrane conformational changes during the isolation procedure.

Interactions between [^{3}H]ADP and [^{35}S]Gum or [^{35}S]Atr

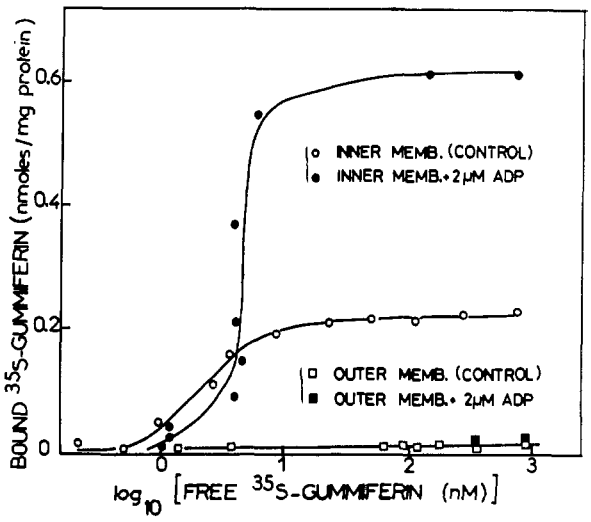

FIGURE 6: Binding of [^{35}S]carboxyatractyloside ([^{35}S]Gum) to isolated inner and outer mitochondrial membranes; effect of ADP. Inner mitochondrial membrane vesicles devoid of matrix (3 mg of protein) or outer mitochondrial membrane vesicles (1.4 mg of protein) were incubated for 45 min at 2° in 10 ml of the standard $MgCl_2$ medium and increasing concentrations of [^{35}S]Gum with or without ADP. For other conditions see Methods.

for Binding to Inner Mitochondrial Membrane. As shown above, ADP translocation is inhibited noncompetitively by Gum and competitively by Atr. Binding experiments to be described now corroborate the kinetic evidence for the two above types of inhibition. The binding assays were carried out with inner mitochondrial membrane vesicles whose properties, as far as adenine nucleotide are concerned, can be summarized as follows (Vignais *et al.*, 1972a). They are largely depleted of endogenous adenine nucleotides (1–1.5 nmol/mg of protein as compared to 10–15 nmol in whole mitochondria). Their endogenous adenine nucleotides which are rapidly exchanged with external ADP are mainly located on the ADP carrier as shown by the following observation. Atr removes part of the bound ADP when added after ADP and prevents the ADP bidding when added simultaneously; the amount of ADP whose binding is prevented by Atr equals the amount of bound ADP removed by Atr.

TABLE IV: Release of Bound [^{35}S]Carboxyatractyloside ([^{35}S]-Gum) and [^{35}S]Atractyloside ([^{35}S]Atr).[a]

Additions	[^{35}S]Gum		[^{35}S]Atr	
	Remaining Bound[b]	% Released	Remaining Bound[b]	% Released
None	1.81	6[c]	0.92	23[c]
0.1 μM Gum	1.62	15	0.65	48
1 μM Gum	1.25	37	0.56	56
1 μM Atr	1.68	12	0.62	51
20 μM Atr	1.44	25	0.51	59
5 μM bongkrekic acid	1.79	7		
100 μM ADP	1.83	6		
5 μM bongkrekic acid + 100 μM ADP	1.15	42		

[a] Rat liver mitochondria (6.6 mg of protein) loaded with [^{35}S]Gum or [^{35}S]Atr were incubated for 2 min at 20° and then 1 hr at 2° in 5 ml of the standard $MgCl_2$ medium, containing either unlabeled Gum or Atr or bongkrekic acid or ADP as indicated. The incubation was stopped by centrifugation at 15,000*g* for 10 min and the radioactivity of the pellet counted by scintillation. [b] mol/mol of Cytochrome *a*. [c] Spontaneous release.

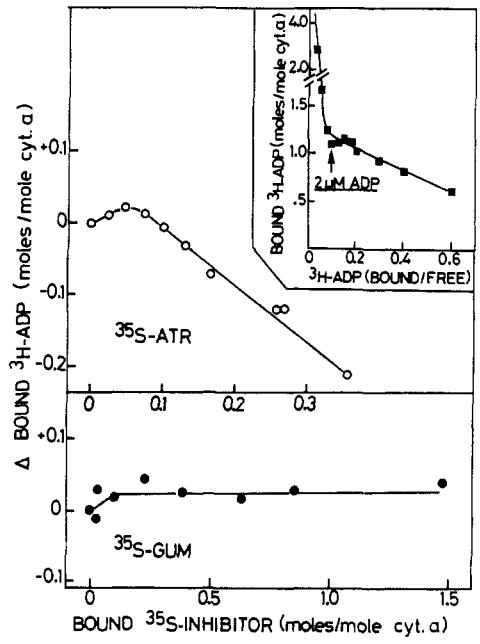

FIGURE 7: Competition between [^{3}H]ADP and either [^{35}S]Gum or [^{35}S]Atr for binding to isolated inner mitochondrial membranes from rat liver. Competition assay: same conditions as described for insert using a fixed concentration of [^{3}H]ADP (2 μM) and increasing concentrations of [^{35}S]Atr and [^{35}S]Gum. For other details see Methods. [^{3}H]ADP binding assay: inner membrane vesicles devoid of matrix (1.9 mg of protein) were incubated for 45 min at 2° in 10 ml of the standard EDTA medium with increasing concentrations of [^{3}H]ADP. The incubation was ended by centrifugation and the radioactivity of the pellet measured by scintillation.

A typical Scatchard plot illustrating the ADP binding to inner membrane vesicles is given in the inset of Figure 7. It can be tentatively decomposed in two slopes of high affinity ($K_d < 0.1$ μM) and low affinity ($K_d > 20$ μM). Interactions between [^{3}H]ADP on the one hand and [^{35}S]Gum or [^{35}S]-Atr on the other were tested at 2 μM ADP. In both cases, inner membrane vesicles were incubated in a series of tubes with a fixed concentration of [^{3}H]ADP (2 μM) and increasing concentrations of [^{35}S]Gum or [^{35}S]Atr. Whereas [^{35}S]Atr competed for binding with [^{3}H]ADP (shown by the fact that the binding of [^{35}S]Atr prevented that of [^{3}H]ADP), [^{35}S]-Gum hardly affected the amount of bound [^{3}H]ADP. The absence of competition between [^{3}H]ADP and [^{35}S]Gum for binding and, on the contrary, the demonstration of a competition between [^{3}H]ADP and [^{35}S]Atr are in good agreement with the role of noncompetitive and competitive inhibitors of ADP translocation played by Gum and Atr, respectively.

Competition between Gum and Atr for Binding to Mitochondria. Although Gum and Atr are both specific inhibitors of the adenine nucleotide translocation in mitochondria, they differ in their inhibitory and binding properties: a sharp

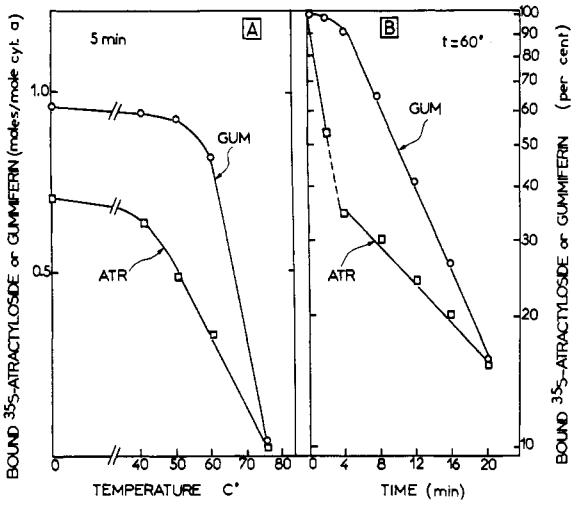

FIGURE 8: Effect of heating on the binding capacity and affinity of rat liver mitochondria for [^{35}S]carboxyatractyloside ([^{35}S]Gum) and [^{35}S]Atr. Rat liver mitochondria in 0.27 M sucrose (29 mg of protein/ml) were heated at different temperatures and for different periods of time as indicated. Aliquots of the mitochondrial suspension (8.7 mg of protein) were incubated with [^{35}S]Gum or [^{35}S]Atr in 10 ml of the standard $MgCl_2$ medium for 45 min at 2° and centrifuged as described in Methods.

saturation is observed for [^{35}S]Gum binding but not for [^{35}S]-Atr binding (Vignais *et al.*, 1971b) and the competition observed between ADP and Atr does not hold in the case of Gum (see above). As shown elsewhere (Vignais *et al.*, 1972a), Gum and Atr compete for binding to mitochondria indicating that their sites are either closely related or the same. The *competitive* inhibition of [^{35}S]Gum binding by Atr contrasts with the *noncompetitive* inhibition of [^{35}S]Gum binding by bongkrekic acid, an antibiotic which inhibits the phosphorylation of added ADP (Welling *et al.*, 1960) by preventing the ADP translocation (Henderson and Lardy, 1970).

The above data were corroborated by experiments showing that [^{35}S]Gum previously bound to mitochondria was released upon addition of Atr and conversely that bound [^{35}S]-Atr was released by added Gum (Table IV). In these displacement experiments, identification of released [^{35}S]Gum and [^{35}S]Atr was obtained by electrophoresis and chromatography. Bound [^{35}S]Gum was displaced by unlabeled Gum, which points to the reversible character of the Gum binding. The same observation holds for bound [^{35}S]Atr. On the other hand, bongkrekic acid and ADP added separately to [^{35}S]-Gum-loaded mitochondria were without effect, but when added together, they induced an extensive release of [^{35}S]-Gum. The synergic effect of ADP and bongkrekic acid is in line with the potentiating effect of ADP on the inhibition by bongkrekic acid of the ADP translocation as reported by Kemp *et al.* (1970).

Modification of the Binding Properties of [^{35}S]Gum and [^{35}S]Atr to Mitochondria by Specific Alteration of Mitochondrial Membrane Components. Treatments used to explore the chemical nature of the Gum binding components in mitochondria were (a) heating of mitochondria before addition of [^{35}S]Gum, (b) enzymatic digestion by proteolytic (trypsin) or lipolytic (phospholipase C) enzymes, (c) photooxidation in the presence of Rose Bengal, and (d) delipidation by aqueous acetone (90%). Most of these tests were performed simultaneously with [^{35}S]Atr and [^{35}S]Gum.

Heating rat liver mitochondria in 0.27 M sucrose–2 mM Tris-

TABLE V: Effect of Trypsin on the Binding of [^{35}S]Carboxytractyloside ([^{35}S]Gum) and [^{35}S]Atractyloside ([^{35}S]Atr) to Rat Liver Mitochondria.[a]

Additions	Treatment by Trypsin	Bound [^{35}S]Gum or [^{35}S]Atr (mol/mol of Cytochrome *a*)
[^{35}S]Gum	−	1.16
[^{35}S]Gum	+	0.97
[^{35}S]Gum + ADP	−	1.15
[^{35}S]Gum + ADP	+	1.05
[^{35}S]Atr	−	0.76
[^{35}S]Atr	+	0.44
[^{35}S]Atr + ADP	−	0.49
[^{35}S]Atr + ADP	+	0.43

[a] Rat liver mitochondria were incubated with trypsin (15 mg of trypsin/100 mg of mitochondrial protein) in 10 ml of the standard EDTA medium for 12 min at 25°. A control was run without trypsin. After addition of 30 mg of trypsin inhibitor, the suspension was centrifuged and the mitochondria resuspended in 0.27 M sucrose. Incubation with [^{35}S]Gum or [^{35}S]Atr was carried out at 2° for 30 min in 10 ml of the standard EDTA medium in the presence of 0.165 μM [^{35}S]Gum or [^{35}S]Atr and 0.2 mM ADP when indicated. The incubation was ended by centrifugation.

HCl, pH 7.4, for 5 min above 45° decreased the subsequent binding of both [^{35}S]Gum or [^{35}S]Atr and was totally inhibitory at 75° (Figure 8A). However, as shown in Figure 8B, heating mitochondria at 60° for 4 min destroyed nearly 70% of the [^{35}S]Atr binding capacity and practically did not alter the [^{35}S]Gum binding efficiency. The time-dependent effect of heating on [^{35}S]Atr binding (Figure 8B) suggests a heterogeneity in the Atr binding sites, which may be related to the occurrence in mitochondria of high affinity binding sites sensitive to ADP and of low affinity binding sites which are ADP insensitive (Vignais *et al.*, 1972a). Experiments not shown here indicated that the Atr sites from which Atr could be displaced by ADP were the most sensitive to heating.

Incubation of mitochondria with trypsin (Table V) resulted in a decreased ability to bind [^{35}S]Gum and [^{35}S]-Atr. As in heating experiments, the ADP-sensitive Atr binding was the most sensitive to trypsin.

Photooxidation of mitochondria, at pH 8, led to a substantial decrease in affinity for [^{35}S]Atr and in the total amount of [^{35}S]Atr and [^{35}S]Gum binding sites (Figure 9). In independent assays, it was observed that no protective effect against photooxidation was conferred by ADP. Since histidine, among other photooxidizable amino acids such as cysteine, methionine, tryptophan, and tyrosine, is more labile to photooxidation at alkaline pH, complementary tests were run at different pH values ranging from 6 to 8.5. No marked pH dependence was found. This, however, does not exclude the involvement of a histidine residue located in a lipophilic environment and remaining unprotonated at pH 6 (see Hoffee *et al.*, 1967). The above set of experiments (heating, digestion by trypsin, photooxidation) demonstrates the involvement of protein in the Gum and Atr binding sites. However, it is clear that the Gum binding is less susceptible to an alteration of the site by trypsin or heat treatment than the Atr binding.

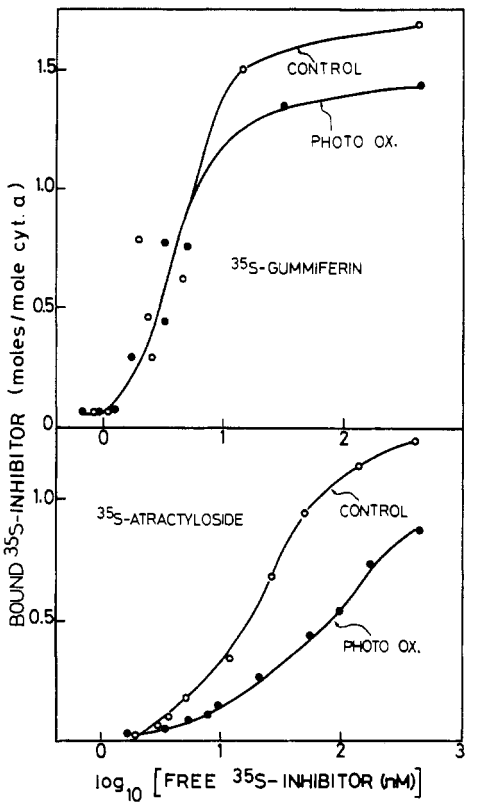

FIGURE 9: [^{35}S]Atr and [^{35}S]carboxyatractyloside ([^{35}S]Gum) binding altered by photooxidation of mitochondria. A 50-ml beaker containing 5 ml of the suspension of mitochondria (42 mg of protein/ml), in 0.27 M sucrose–0.01 M Tris-HCl (pH 8.0)–10^{-5} M Rose Bengal, was placed in crushed ice and covered with a petri dish filled with water to a height of 2–3 mm. A piece of tubing was inserted between the beaker and the petri dish to allow air to bubble through the mitochondrial suspension. Irradiation was performed for 5 min by means of two 250-W tungsten lamps located side by side at 20 cm above the bottom of the vessel. Aliquots (200 μl, 8.4 mg of protein) of the mitochondrial suspension were incubated in 10 ml of 110 mM KCl–0.1 mM EDTA–10 mM Tris-sulfate, pH 8.0. for 45 min at 2° with increasing amounts of [^{35}S]Gum or [^{35}S]-Atr.

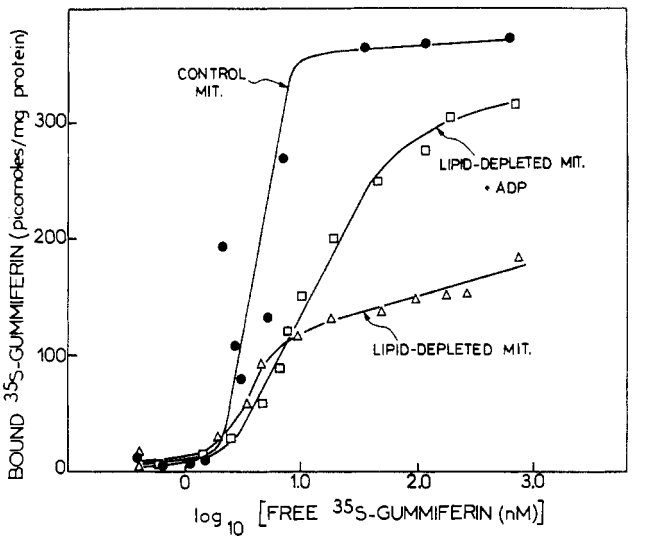

FIGURE 10: Effect of lipid depletion on the binding capacity and affinity of rat liver mitochondria for [^{35}S]carboxyatractyloside ([^{35}S]Gum). Aliquot fractions (100 μl, 6 mg of protein) of lipid-depleted mitochondria (see Methods) were incubated in 10 ml of the standard $MgCl_2$ medium, increasing amounts of [^{35}S]Gum, and, where present, 100 μM ADP. For other conditions see Methods. Note that bound [^{35}S]Gum is referred to protein instead of cytochrome *a* content because of alteration of the cytochrome *a* spectrum in lipid-depleted mitochondria.

The mitochondrial lipids also play a role in Gum and Atr binding. Removal of lipids from mitochondria by aqueous acetone resulted in a striking decrease of the total amount of Gum binding sites and of the cooperative interactions observed for Gum binding (Figure 10). Addition of 100 μM ADP to lipid-depleted mitochondria nearly completely restores the Gum binding capacity at saturation, but not the loss of cooperativity, a result which suggests the implication of mitochondrial lipids in the cooperative process of Gum binding. Preliminary experiments carried out with phospholipase C have led to results essentially similar to those obtained with the aqueous acetone treatment. Comparative experiments carried out with [^{35}S]Atr showed that [^{35}S]Atr binding was virtually totally lost upon lipid depletion of mitochondria.

Interestingly, mild sonication of mitochondria modified the titration curves for Gum and Atr, with a complete loss of cooperative interactions as illustrated by the Scatchard representation in Figure 11. This finding supports the concept of oligomeric nature of the Gum (and most likely Atr also) binding sites. However, the total number of sites for both inhibitors was not altered. At variance with our data on Atr binding, Klingenberg *et al.* (1971, 1972) using a single (saturating) concentration of Atr (20 μM) found that sonication doubled the amount of bound Atr. They concluded that, although Atr is a nonpenetrant inhibitor in whole mitochondria, Atr binding sites are present both on the inner and the outer surfaces of the inner mitochondrial membrane, a result fitting with their assumption of identical sites for ADP and Atr.

Binding and Inhibitory Properties of apo-[^{35}S]Gum and apo-[^{35}S]Atr. Alteration of different chemical groups in Gum (desulfatation, acetylation, tritylation, deacylation) resulted in less potent inhibitors. Among them, apo-Gum, obtained by removal of the isovaleryl residue (see Methods), deserves more attention since its behavior recalls that of Atr in several ways. The inhibition of the ADP-stimulated respiration caused by apo-Gum is relieved by increasing the concentration of ADP. On the other hand, apo[^{35}S]Gum binding does not display cooperative interactions as Gum does; furthermore, ADP decreases the apo-Gum binding affinity, whereas it has no effect or a slight stimulatory effect on the cooperative feature of the Gum binding (Figure 12A). The removal of the isovaleryl group from Atr resulting in apo-Atr led also to a decrease in the binding affinity and in the inhibitory efficiency. As for [^{35}S]Atr and for apo-[^{35}S]Gum, the affinity of apo-[^{35}S]Atr for mitochondria is decreased by excess ADP (Figure 12B).

apo-[^{35}S]Gum binding was prevented competitively by Atr but unexpectedly noncompetitively by Gum (Figure 13). In other experiments not shown here, it was found that bongkrekic acid also inhibits the apo-[^{35}S]Gum binding noncompetitively as it does for [^{35}S]Gum. A brief summary of observed interactions between [^{35}S]Gum, apo-[^{35}S]Gum, and [^{35}S]Atr for binding to mitochondrial membrane is given in Table VI.

Discussion

Data presented in this paper demonstrate that Gum is a specific, noncompetitive inhibitor of the adenine nucleotide

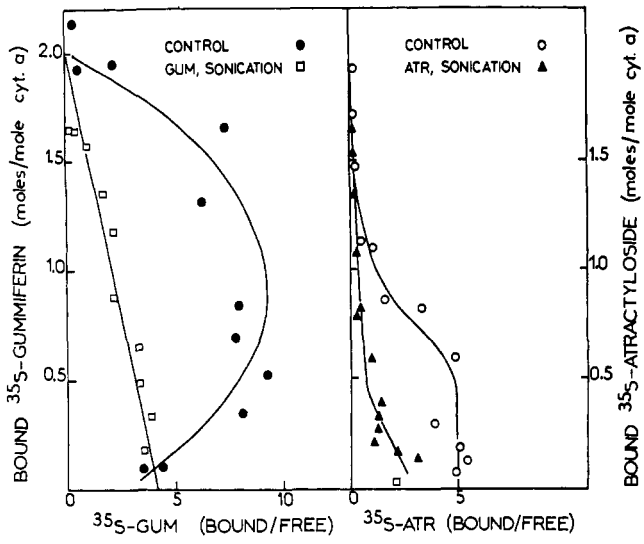

FIGURE 11: Effect of sonication on the binding capacity and affinity of rat liver mitochondria for [^{35}S]carboxyatractyloside ([^{35}S]Gum) and [^{35}S]Atr. Identical aliquot fractions of the mitochondrial suspension (8 mg of protein in 200 μl of 0.27 M sucrose) were distributed in a set of beakers containing 10 ml of the standard $MgCl_2$ medium and increasing concentrations of [^{35}S]Gum or [^{35}S]Atr. After 5 min of incubation at 2° they were exposed to sonic oscillations for 30 sec at 2–4° (Branson sonifier, maximal output) and then left to stand for another 40-min period before centrifugation at high speed (30,000g for 30 min). Parallel control assays were run with mitochondria not submitted to sonication. The radioactivity of the pellets was counted by scintillation.

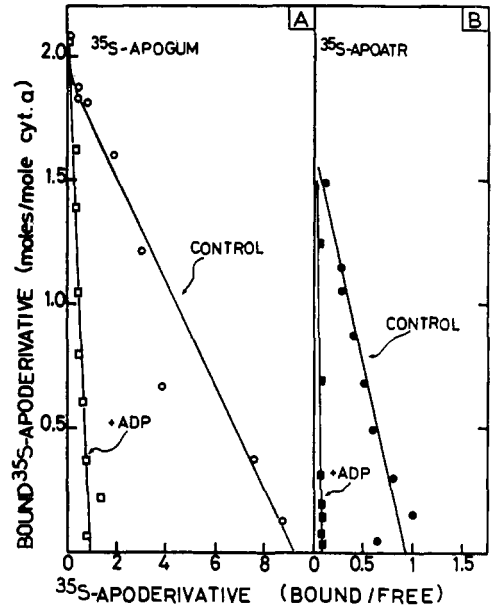

FIGURE 12: Binding of [^{35}S]apocarboxyatractyloside (apo-[^{35}S]Gum) and apo-[^{35}S]Atr to rat liver mitochondria; effect of ADP (200 μM). The binding assays were carried out in 10 ml of standard $MgCl_2$ medium with 6.2 mg of mitochondrial protein and increasing concentrations of apo[^{35}S]Gum or [^{35}S]Atr.

translocation in mitochondria. It can therefore be added to the list of other potent inhibitors, either competitive, like Atr and long-chain fatty acyl-CoA (Pande and Blanchaer, 1971; Vaartjes *et al.*, 1972) or noncompetitive, like bongkrekic acid (Henderson and Lardy, 1970). The high specificity of the inhibition caused by Gum, as well as its noncompetitive character with respect to ADP and the absence of a lag period for maximal action, make Gum a more suitable tool than Atr, long-chain acyl-CoA, or bongkrekic acid to stop incubation in kinetic assays of adenine nucleotide translocation.

Although Gum and its derivatives Atr and apo-Gum probably share at least part of the same site as shown by mutual exclusion for binding between Atr and Gum and between apo-Gum and Atr, they differ in complementariness to this common site, as reflected by differences in their binding properties. For instance, the presence of a supplementary carboxylic group at C_4 of the genin moiety in Gum with respect to Atr results in enhancement of interaction with the translocase, as evidenced by the following observations: (1) the inhibition of ADP translocation by Gum is noncompetitive while that caused by Atr is competitive; (2) at neutral pH Gum binding displays strong cooperative interactions up to saturation in contrast to Atr; (3) ADP (or ATP) decreases the Atr affinity for *intact mitochondria* whereas it has no effect on or sometimes increases the cooperative interactions for Gum binding. This points to the importance of the genin moiety in site recognition, corroborating an earlier conclusion (Vignais *et al.*, 1966) that atractyligenin is the active moiety of Atr. In those studies (Vignais *et al.*, 1966) the role played by the

TABLE VI: Summary of Inhibitor Interactions.

Ligand	Inhibitor	
	Competitive	Noncompetitive
[^{35}S]Gum	Atr	Bongkrekic acid (+ ADP[a])
apo-[^{35}S]Gum	Atr ADP	Gum Bongkrekic acid
[^{35}S]Atr	ADP Gum	Bongkrekic acid

[a] ADP does not inhibit [^{35}S]Gum binding but strongly potentiates the inhibition by bongkrekic acid.

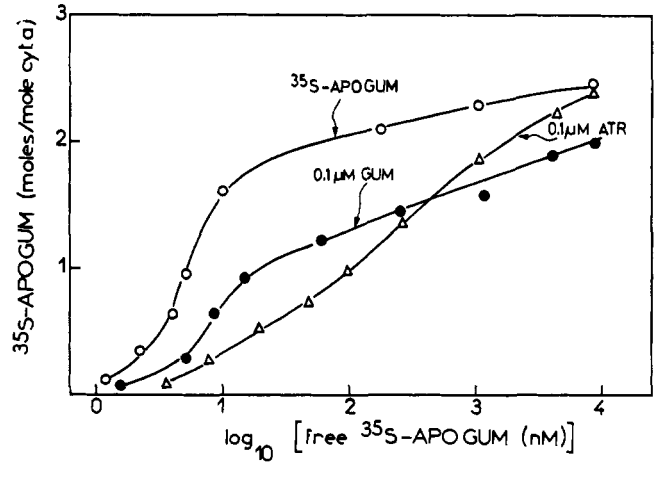

FIGURE 13: Prevention of [^{35}S]apocarboxyatractyloside (apo-[^{35}S]Gum) binding to rat liver mitochondria by Gum or Atr. The binding assay was carried out as described in Figure 12 with 8.4 mg of protein.

4-carboxylic group of the atractyligenin molecule had already been stressed. For instance, atractylitriol, obtained by reduction of the 4-carboxylic group into alcohol, did not inhibit the ADP translocation nor did other atractyligenin analogs of the kaurenolide series in which the carboxylic group was in the form of internal lactone. It was also shown that when the second substituent at the C_4 position of the diterpene was a methyl, as in steviol, the inhibition of ADP translocation was noncompetitive with ADP. Therefore, the known inhibitors of the adenine nucleotide translocation belonging to the Atr family can be divided into two groups, according to whether they are competitive inhibitors with respect to ADP (Atr, atractyligenin, and apo-Gum) or noncompetitive inhibitors (Gum, steviol, dihydrosteviol).

Another example of structural complementariness to the translocase, added in this paper, is the effect of removing the isovaleryl group. The isovaleryl group most likely stabilizes the Gum molecule in a conformation close to the one shown by the model in Figure 1 where hydrophobic interactions between the isovaleryl group and the diterpene moiety are maximal. apo-Gum, which lacks the isovaleryl group, behaves differently from Gum and more like Atr in that it competitively inhibits the ADP translocation and it competes with ADP for binding to mitochondria. Furthermore, apo-Gum binding is inhibited competitively by Atr but noncompetitively by Gum.

The acidic strength of the carboxyl groups may also play an important role in the binding properties of Gum and its derivatives. In preliminary studies we have determined (unpublished results) the pK values of those carboxyl groups and found for Gum, pK_1 = 3.4 and pK_2 = 6.7; for apo-Gum, pK_1 = 4.0 and pK_2 = 5.7, for Atr, pK = 5.7; for apo-Atr, pK = 5.7. The high pK_2 value for Gum indicates that the second carboxyl group may be stabilized by H bonding either with the first carboxyl or with the carbonyl group of the ester bond at the C_2' position of glucose. In dicarboxylic acids, in general, the closer the carboxyl groups, the greater the difference between the first and second ionization constants (Eliel, 1962). In Gum and apo-Gum, stabilization may occur by hydrogen bonding between the two carboxyl groups. However, the large difference between the pK values of the carboxyl groups in Gum (3.3 pH units) as compared to apo-Gum (1.7 pH units) and the high value of pK_2, 6.7, for Gum compared to 5.7 for apo-Gum point to modifications of acid strength resulting from the removal of the isovaleric acid. Thus, in Gum, there may also be interactions stabilized by H bonding between the carbonyl group of the isovaleric ester and one carboxylic group. A favored conformation endowing Gum with its specific binding properties can be brought about in this way. In apo-Gum, a greater mobility of the glucose moiety with respect to the diterpene moiety due to the absence of the isovaleryl residue would not allow the stabilization of such a conformation of the whole molecule.

By maneuvering the SASM model[3] of Gum it was observed that the axial carboxyl group (which is the one found in Atr) can easily come quite close to the carbonyl group of the ester bond at the C_2' position of the glucose. The equatorial carboxyl of Gum should be stronger because the corresponding anion is more readily solvated than the other one, which, being axial and close to the C_{10} methyl, encounters some steric hindrance to solvation. It is inferred that in Gum the axial carboxyl has a pK of 6.7 and the equatorial carboxyl a pK of 3.3. In apo-Gum the axial carboxyl would have a pK of 5.7 (similar to the pK found in Atr).

Turning to the problem of the topology of the Gum binding sites one may ask the following questions. (1) Are there Gum binding sites on both sides of the inner membrane or only on the outer side of it? (2) How much of the membrane surface is occupied by Gum and what is the distribution of those sites on the inner membrane? (3) What are the molecular components of the Gum binding site and are there any special geometric features of the binding site which favor Gum binding? (4) What are the relationships between the Gum sites and the ADP binding sites?

The location of the Gum binding sites with respect to the outer and inner sides of the inner mitochondrial membrane has been tentatively investigated in sonication experiments. It is currently accepted that sonication of mitochondria leads to an inversion of the polarity of the inner mitochondrial membrane. Assuming that Gum is a nonpenetrant inhibitor, the occurrence of Gum binding sites both on the outer and on the inner surfaces of the inner mitochondrial membrane should result, upon sonication of mitochondria in the presence of Gum, in an increase of the number of binding sites. Experimental data presented in this paper showing no effect of sonication on the number of sites for Gum or Atr do not support that possibility but rather suggest that the Gum binding sites are distributed essentially on the outer surface of the inner mitochondrial membrane.

A rough estimate of the surface of the inner membrane of rat liver mitochondria occupied by the Gum sites can be made. Assuming a value of 4.3×10^9 mitochondria per milligram of mitochondrial protein (Gear and Bednarek, 1972), a total surface of the order of 16 μ^2 for the inner membrane including cristae (Lehninger, 1964), and a projected area of about 200 Å^2 for the Gum molecule it can be calculated that 0.5–0.7% of the area of the inner mitochondrial membrane is occupied by Gum at saturation.

The central role played by proteins among the components of the Gum site is evidenced by the decrease in binding affinity and capacity upon trypsin digestion, heat denaturation, or photooxidation and by the specificity of the binding reaction toward Gum and structurally related compounds. Association of Gum sites in clusters is suggested by the cooperative character of the Gum binding. In line with this assumption, sonication which is likely to disrupt oligomeric structures leads to the disappearance of cooperative Gum binding. A possible change of conformation of protein receptor consequent upon binding Gum may explain why, in spite of rather close affinities for Gum and Atr (K_d values in the range of 5–15 nM), bound [^{35}S]Gum is much less easily displaced by Atr than bound [^{35}S]Atr is by Gum (Table IV). That lipids may be necessary to constrain the protein component(s) of the Gum site in a conformation favoring clusters is plausible in view of the loss of cooperative interactions upon lipid depletion.

In earlier papers the conclusion that the Atr sites are on the ADP translocase or in its close neighborhood was drawn from the finding that Atr and ADP compete for binding (Vignais *et al.*, 1970). Several data allow one to extend this conclusion to Gum, namely the mutual exclusion of Atr and Gum for binding to mitochondria and the interactions between ADP and Gum binding as revealed by the ADP- or ATP-induced restoration of Gum sites in isolated inner mitochondrial membranes which had lost part of the Gum binding capacity.

[3] SASM models are from SASM, 75011 Paris, France.

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Added in Proof

An interaction between the carboxyl groups and the isovaleryl residue in Gum as suggested in the discussion is corroborated by recently measured nuclear magnetic resonance spectra (220 MHz) of atractyloside and gummiferin methyl esters in deuterated dimethyl sulfoxide; the resonance lines of the methyl groups of the isovaleric chain which appear as a doublet (0.89 ppm, $J = 7$ Hz) in atractyloside are split in gummiferin (0.86 ppm, $J = 7$ Hz and 0.89 ppm, $J = 7$ Hz).

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